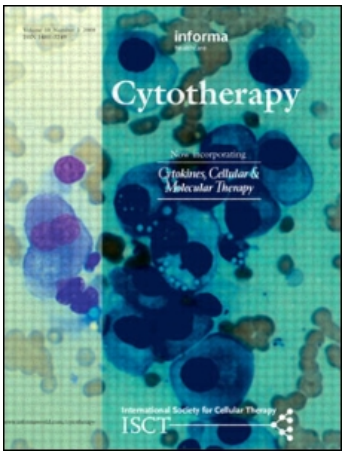

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In vitro expression of erythropoietin by transfected human mesenchymal stromal cells

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Background

Mesenchymal stromal cells (MSC) are pluripotent progenitor cells that can be found in human bone marrow (BM). These cells have low immunogenicity and could suppress alloreactive T-cell responses. In the current study, MSC were tested for their capacity to carry and deliver the erythropoietin (EPO) gene in vitro.

Methods

Expanded BM MSC was transfected with EPO-encoded plasmid pMCV1.2 and EPO-encoded MIDGE (minimalistic immunologically defined gene expression) vector by electroporation. The expressed EPO was used to induce hematopoietic stem cells (HSC) into erythroid colonies.

Results

The results showed that the MIDGE vector was more effective and stable than the plasmid (pMCV1.2) in delivering EPO gene into MSC. The supernatants containing EPO obtained from the transfected cell culture were able to induce the differentiation of HSC into erythroid colonies.

Discussion

MSC hold promise as a cell factory for the production of biologic molecules, and MIDGE vector is more effective and stable than the plasmid in nucleofection involving the EPO gene.

Keywords

erythropoietin, lipofection, mesenchymal stromal cells, minimalistic immunologically defined gene expression (MIDGE), nucleofection, plasmid.

Introduction

Mesenchymal stromal cells (MSC) are adult human pluripotent progenitor cells found in bone marrow (BM). MSC are suitable for manipulation in gene delivery because (1) they are easily isolated and expanded in culture; (2) they are able to maintain an undifferentiated state unless exposed to certain differentiation stimulators and thus can be kept in large volumes for a long period; (c) genetically altered MSC can also be easily recovered after installation *in vivo*; and (d) transduced MSC and their progeny can express newly introduced genes in a less restrictive fashion than other cells, thereby expanding their potential application in treating medical disease. Previous researches have also shown that they have low immunogenicity and even suppress allogeneic T-cell responses [1–4]. Thus allogenic MSC can survive in the microenvironment after *in vivo* transplantation in animal models.

MSC had been used experimentally to carry and deliver numerous therapeutic genes, for example coagulation factor VIII (FVIII) [5], cytotoxic T-lymphocyte associated antigen immunoglobulin (CTLAIg) [6] and α-galactosidase A [7] to treat hemophilia A, graft vs. host disease (GvHD) and Fabry's disease, respectively. Recently MSC have also been shown to have high tumor tropism and were demonstrated to exert an anti-tumor effect and further

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prolong the survival of a rat glioma model when genetically engineered MSC expressing interleukin-2 (IL-2) were injected intratumorally [8,9].

Despite a promising future for gene therapy by manipulating MSC, vector systems for gene therapy strategies should offer both a means of successful transfection and a maximum of safety for the patients. Most gene transfer protocols have used murine replication-defective retroviral vectors or adenoviral vectors. Although retroviral vectors can effectively transduce and integrate into the genome of targeted cells, the risk of oncogene activation has to be considered. Adenoviral vectors have significantly improved transduction efficacy but the death of an 18-year-old patient who received an adenoviral-delivered therapeutic gene in 1999 has raised concern regarding the safety of the therapy using viral vectors [10–12].

Plasmid-based gene transfer using physical or chemical transfection methods avoids these risks. However, transfection efficiency is usually lower and protein expression may not be sustainable for non-viral vectors. There is a substantial risk of immunologic side-effects, including elimination of transfected cells by the host's immune reaction when therapeutically unwanted eu- and prokaryotic proteins (e.g. antibiotic resistance genes, viral protein genes and prokaryotic promoters) are expressed as antigen (Ag) on transfected cells [13]. Moreover, plasmid DNA contains immune stimulatory sequences, called CpG motifs, and they can activate both the innate and acquired arms of immune responses [14,15].

The construct of minimalistic immunologically defined gene expression (MIDGE) has been described previously and has abolished the transfer of therapeutically detrimental sequences [13]. Hence in this study we wanted to determine the capability of MSC to carry and express therapeutic genes using MIDGE as a vector for gene delivery *in vitro* by means of electroporation. The gene we chose for the study was erythropoietin (EPO), which is important in stimulating the production of red blood cells (RBC). The MSC used had been isolated and identified morphologically, cytochemically and immunochemically by flow cytometry. The cells were capable of differentiating into adipocytes, chondrocytes and osteoblasts [16,17].

Methods

Samples

Two samples of human MSC were used in the gene transfer study. The first sample was labeled as pMSC and was isolated from the BM aspirate of a megaloblastic anemia patient, who came for a routine check up at the Hospital Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia, after informed consent and under a protocol approved by the UKM Research Committee and Ethics Committee. The second human MSC sample was bought from Cambrex Bio Science Walkersville Inc. (Walkersville, MD, USA) and labeled as hMSC.

Isolation of BM MSC

Five milliliters of BM aspirate were layered on top of 3 mL Ficoll–Pague (Amersham Biosciences, Uppsala, Sweden) and centrifuged at 400 ***g*** for 30 min. The mononuclear cells (MNC) in the interface (density gradient 1.077 g/L) were extracted and washed twice with culture medium by centrifuging at 100 ***g*** for 10 min. The pellet cells were then suspended in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, New York, NY, USA) and the viability of cells counted by hematocytometer and trypan blue staining. The results showed that the percentage of viable cells was 99.7%. The cells were then seeded at a density of 1×10^7 cells in a 25-m^2 plastic flask containing DMEM supplemented with 10% fetal bovine serum (FBS; Gibco). The flask was then incubated in 5% CO_2 in air and monitored daily. Once the cells reached confluency, they were detached with 1 mL 0.25% trypsin–EDTA (Gibco) and replated again into new flasks at a similar cell density. Characterization of DMEM-derived adherent cells was performed by using cells from the third and fourth passages 4–5 weeks after the initial culture [16,17].

Construction of MIDGE-EPO

First-strand cDNA was synthesized by using fetal liver total RNA (Cell Applications Inc., San Diego, CA, USA) as template and oligo dT as synthesis primer. Reverse transcription was performed using SuperScript III RNase H-Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Polymerase chain reaction (PCR) amplification was then carried out using this first-strand cDNA synthesis product as template and synthetic oligonucleotides upstream containing a *Bam*HI restriction site (5′-GCGAGCTC-CACCATGGGGGTGCACGAATGTCCTGCC-3′) and downstream containing a *Sac*I restriction site (5′-GAGCTCTCATCTGTCCCCTGTCCTGCAGGC-3′) targeted at the 5′-end and 3′-end of the EPO gene in the following PCR reaction: 30 cycles of amplification (94°C,

15 s; 58°C, 45 s; 72°C, 30 s) using a LA Taq PCR mixture (Takara Bio Inc., Otsu, Shiga, Japan) and Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany).

Plasmid pMCV was obtained from Mologen (Berlin, Germany) as a source plasmid for synthesis of the MIDGE vectors. The full-length EPO gene was then cloned into the plasmid via the *Bam*HI and *Sac*I restriction sites. The pMCV encoded EPO was then cleaved with *Esp*3I (Fermentas, Vilnius, Lithuania) to produce double-stranded DNA molecules consisting solely of the expression cassette. The expression cassette was then ligated with an oligonucleotide hairpin structure at both ends of the double-stranded DNA to produce the MIDGE vector containing the EPO gene (MIDGE-EPO). The sequences of the hairpin used at the 5′- and 3′-end of the expression cassette were GCGTCTTTTGACGCAGGG and AGGGCGCAGTTTTCTGCG (Sigma Proligo, Singapore).

Nucleofection of human MSC with MIDGE-EPO or pEGFP

Nucleofection of human MSC was done using a human MSC nucleofector kit and nucleofector (Amaxa GmbH, Cologne, Germany). Prior to nucleofection, a Petri dish culture containing 1.0 mL DMEM was incubated in a CO_2 incubator at 37°C. Early passage of MSC (P3) was trypsinized with trypsin–EDTA and neutralized with DMEM supplemented with 10.0% FBS. The cell number was then counted by trypan blue staining with a hematocytometer. A number of 1.25×10^5 cells was then aliquoted into an Eppendorf tube and centrifuged at 200 ***g*** for 10 min. The pellet was then added to 100 µL nucleofector solution and 2.0 µg MIDGE-EPO, plasmid pMCV1.2-EPO or plasmid EGFP (pEGFP). The mixture was resuspended slowly and transferred into a cuvette and inserted into the nucleofector. The program U-23 was chosen for high transfection efficiency. After transfection, the mixture in the cuvette was added to 500.0 µL DMEM and transferred gently into a prepared Petri dish culture. The Petri dish culture was then incubated in the CO_2 incubator at 37°C and monitored daily. For a no-DNA control, distilled water was used to replace the vector expression systems. A no-transfection control was also carried out by not pulsing the cells with any transfection program.

Determination of EPO expression by ELISA

All the supernatant from the transfected and non-transfected cells (from the no-transfection control) were collected at various time points and changed with new medium, i.e. DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. The collected supernatant was used for EPO expression determination according to the procedures recommended by the manufacturer (human erythropoietin ELISA immunoassay kit, Stem Cell Technologies, Vancouver, Canada). A background control with culture medium alone was also performed.

Differentiation of $CD34^+$ enriched cells into erythroid colonies

MegaCult-C collagen and medium without cytokines kit, human recombinant EPO for positive control, stem cell factor and MegaCult-C staining kit for erythroid colonies were obtained from Stem Cell Technologies. $CD34^+$ enriched cells were obtained from a normal apheresis donor after informed consent and under a protocol approved by the UKM Research Committee and Ethics Committee. Recombinant EPO or supernatant containing the highest EPO level from pMCV1.2-EPO- and MIDGE-EPO- transfected cells and non-transfected cells were added with stem cell factor to induce the differentiation of $CD34^+$ cells into erythroid colonies. Collagen was mixed with the cell medium suspension and the suspension containing 1500 cells was then subsequently dispersed into a single-chamber culture slide with a 22-G blunt-ended syringe. After 2 weeks the gels were dehydrated and fixed. Immunocytochemical staining was then performed using glycophorin A antibody (Ab) directed against human erythroid colonies (CFU-E and BFU-E) and the imunoreaction was detected with an alkaline phosphatase detection system. For a negative control, Ab anti-trinitrophenol (anti-TNP) was used. The staining protocol was carried out according to the recommendations in the MegaCult-C staining kit for erythroid colonies.

Results

Isolation and identification of MSC from human BM aspirates

Isolated MNC from the BM adhered as fibroblast-like cells (Figure 1). These cells propagated rapidly and, when cells at P3 were immunophenotyped, they showed abundant expression of CD10, CD13, CD29, CD44, CD73, CD90, CD105 and CD147. However, the cells did not express

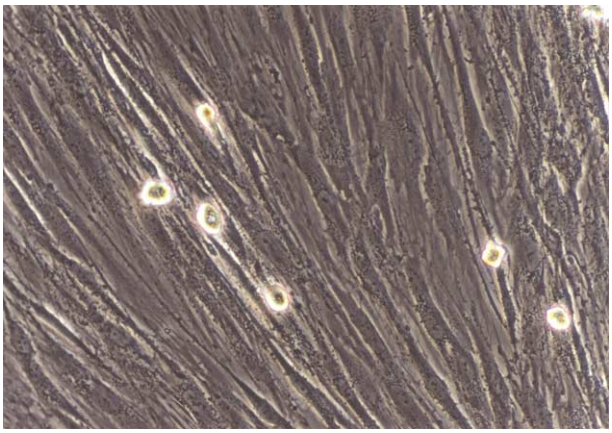

Figure 1. The isolated cells were fibroblast-like and they showed a high proliferative rate in culture (50 ×).

CD3, CD11c, CD14, CD34, CD45 and HLA-DR, indicating no presence of contaminating hematopoietic cells. These cells were positive to cytochemical staining with periodic acid schiff (PAS) and α-naphtyl acetate esterase (NSE) but not to naphtol AS-D chloroacetate esterase (NASDA), sudan black B (SBB) and alkaline phosphatase stains (ALP). When incubated in specific culture conditions, the fibroblast-like cells were capable of differentiating into adipocytes, chondrocytes and osteoblasts. The differentiation into these cell types were confirmed by Oil Red O, Alcian Blue-PAS and Alizarin Red S stain, respectively, and reverse transcription PCR for mRNA-specific genes for adipogenesis, chondrogenesis and osteogenesis (data not shown).

Construction of plasmid pMCV1.2 and MIDGE vector encoding EPO gene

The EPO gene was successfully cloned from human fetal liver total RNA. The EPO gene was successfully inserted into the multiple cloning site (MCS) of pMCV1.2 and the encoded plasmid was largely expanded in LB broth and extracted for the following transfection experiment and construction of MIDGE vector (Figure 2).

Determination of EPO expression from the transfected MSC

The nucleofection was efficient to deliver pEGFP with more than 50% of the pMSC and hMSC expressing the green fluorescence twenty four hours following nucleofection (Figure 3).

EPO expression was high on day 1 in pMCV-EPO-transfected pMSC and hMSC, respectively, i.e. 4779.40 and

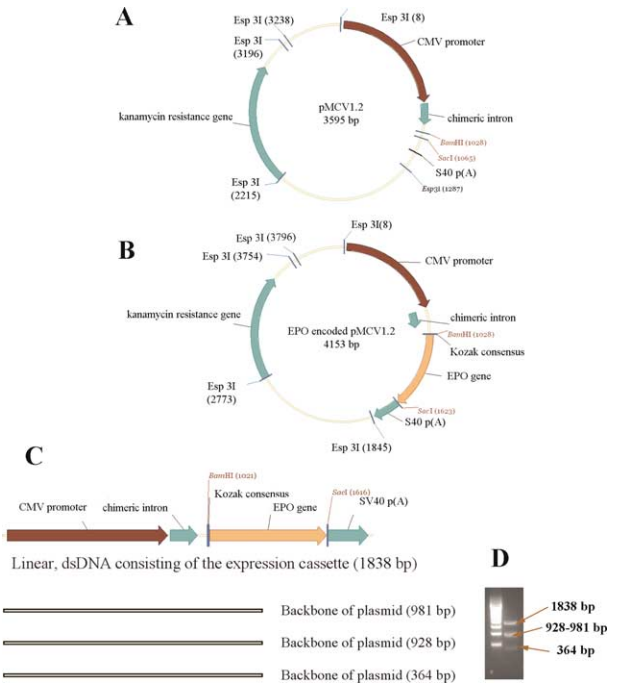

Figure 2. Construction of a MIDGE vector encoded with EPO. (A) Plasmid pMCV1.2. (B) The EPO gene was inserted into the plasmid pMCV1.2. (C) The plasmid was cut with Esp*3I, resulting in a dsDNA consisting of the expression cassette (1838 bp) and three backbones of plasmid (sizes ranged from 364 to 981 bp). (D) The expression cassette was separated from the backbones by electrophoresis on agarose gel. It was then isolated and ligated to the hairpin (data not shown).*

3965.35 mU/mL/1.0 μg vector/1×10^5 cells. On day 6, EPO expression in pMCV-EPO-transfected pMSC rose to 5147.45 mU/mL. Shortly after that EPO expression fell sharply, to less than 30 mU/mL/1.0 μg vector/1×10^5 cells on day 22, and continued to drop, to 5.25 mU/mL/1.0 μg vector/1×10^5 cells 3 months post-transfection. In hMSC transfected with pMCV-EPO, EPO expression fell rapidly to 2.45 mU/mL/1.0 μg vector/1×10^5 cells after day 23. Although the expression was low in these two samples, EPO was still expressed. Meanwhile, in the MIDGE-EPO-transfected hMSC, expression of EPO rose to its peak on day 6, reaching 2683.76 mU/mL/1.0 μg vector/1×10^5 cells. Over the following period of 3 months, EPO expression was surprisingly well maintained, at 641.56 mU/mL. In MIDGE-EPO-transfected pMSC, the highest expression was achieved on day 2 (373.53 mU/mL/1.0 μg vector/1×10^5 cells) and continued to fluctuate to 470.80 mU/mL on day 29 (Figure 4). The discrepancy in initial EPO expression by both MIDGE-transfected

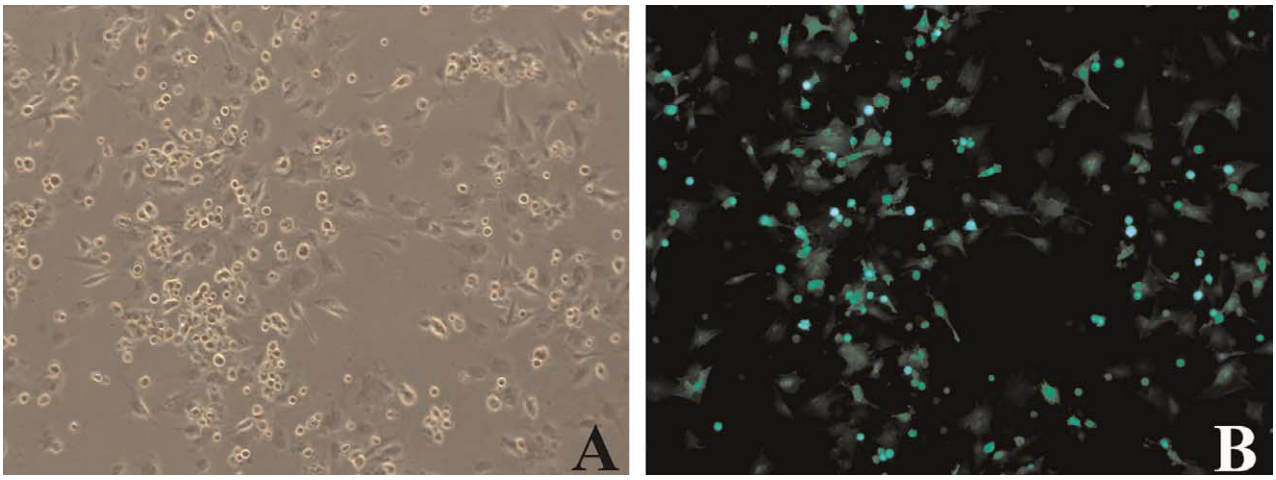

Figure 3. *Twenty-four hour post-nucleofection cells were analyzed by phase-contrast (A) and fluorescence microscopy (B).*

samples might have been because of the discrepancy in transfection efficiency run in two different nucleofection experiments (*c.* 80% and 50%) (data not shown). Meanwhile, the background control and non-transfected cells showed no EPO expression at all time points of measurement.

Differentiation of hematopoietic stem cells ($CD34^+$) into erythroid colonies

Of 1500 cells being seeded onto each chamber slide, the supernatant collected from the EPO-encoded pMCV1.2 and MIDGE vector-transfected cell cultures was found to induce 17.5% and 16.6% of the cells to differentiate into erythroid colonies. The positive control with recombinant EPO could induce 21.4% while the culture of hematopoietic stem cells (HSC) with supernatant from non-transfected cells did not result in any erythroid colony at all. The erythroid colonies were confirmed by performing a specific immunostain for anti-glycophorin A. The colonies were CFU-E, as they had more than 3 but fewer than 200 cells. The staining was performed well, as the colonies did not pick up any red stain at all when anti-TNP, a negative control Ab, was used (Figure 5).

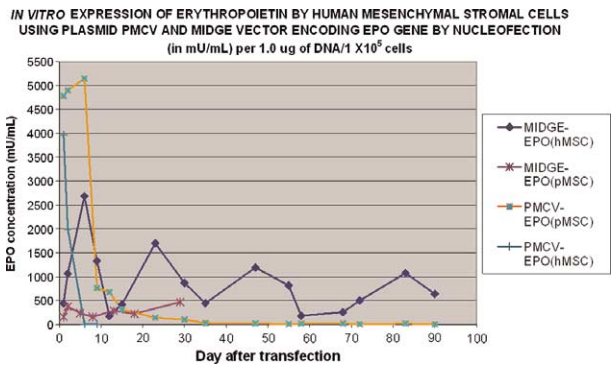

Figure 4. In vitro *expression of EPO by human MSC using pMCV1.2 and MIDGE vector encoding EPO gene by nucleofection. Two samples of MSC were used (labeled as pMSC and hMSC).*

Discussion

We have previously isolated fibroblast-like cells from the BM aspirate of non-malignant patients (Figure 1). These cells showed morphologic and immunophenotypic properties similar to MSC. When stained cytochemically, they showed positive reactivity to PAS and NSE, but not to NASDA, SBB and ALP, indicating that they had glycogen and α-naphtyl acetate esterase, respectively, and were devoid of naphthol AS-D chloroacetate esterase, lipid and alkaline phosphatase, respectively. Fernández *et al.* [18] had shown that MSC isolated from breast cancer patients were positive to PAS and stained weakly with SBB and ALP. Meanwhile, Erices *et al.* [19] had isolated MSC from umbilical cord blood and these fibroblast-like cells demonstrated cytochemical staining results consistent with ours. Besides morphologic, cytochemical and immunophenotypic features, these cells have also been shown to differentiate into adipocytes, chondrocytes and osteoblasts [16,17], a characteristic property of MSC.

The constructed MIDGE vector encoding the EPO gene (approximately 600 bp) was transfected into the human MSC via nucleofection (Figure 2). On day 1 post-nucleofection, the nucleofected MSC expressed 439.22 and 156.99 mU/mL EPO extracellularly/1.0 μg MIDGE-EPO/1×10^5 cells in two different experiments. The

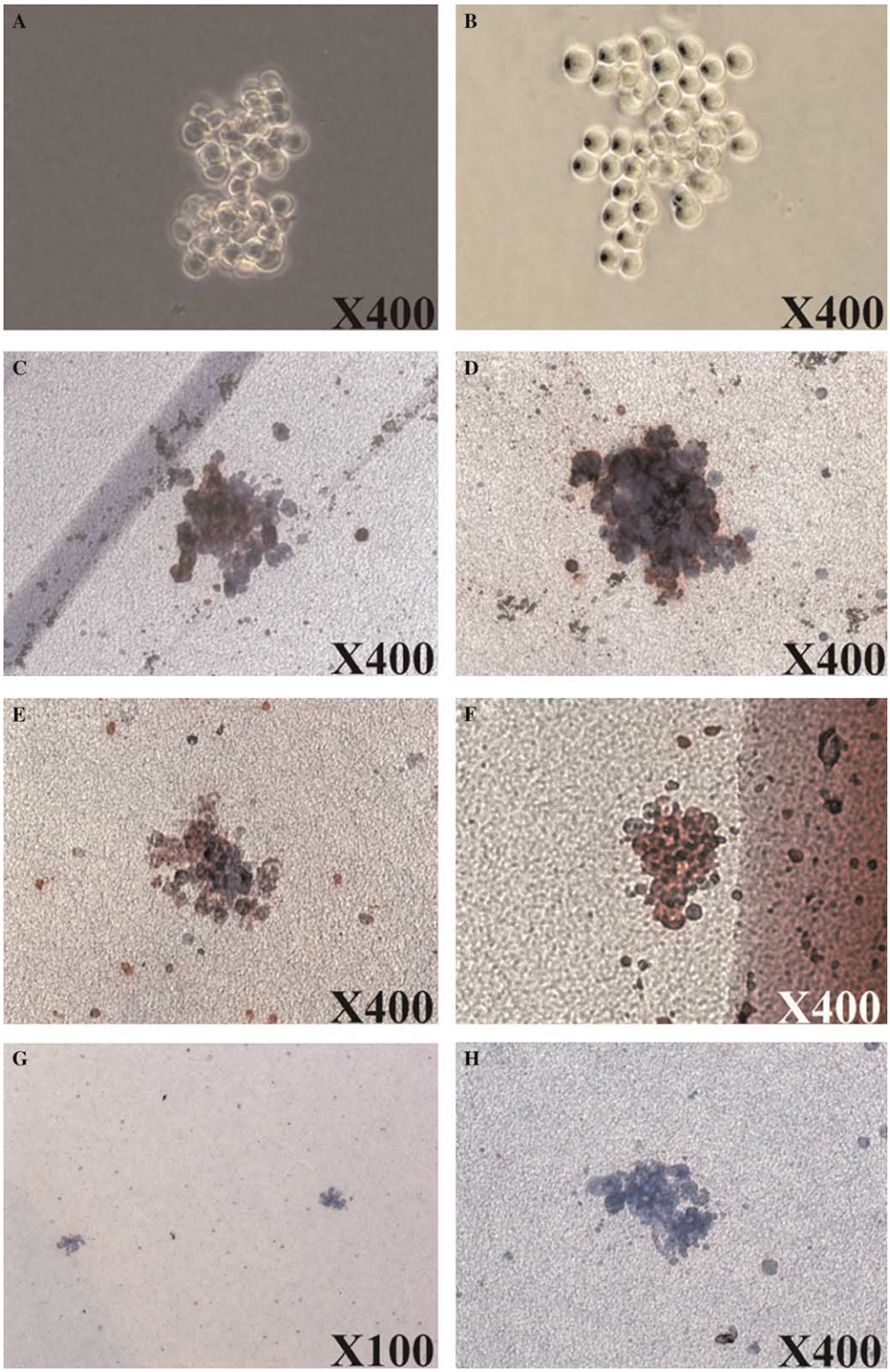

Figure 5. *CFU-E images taken with a phase-contrast microscope. The differentiation of HSC was performed by incubating 3.3 × 10^4 cells with 3 U/mL rhEPO (A) or supernatant collected from the nucleofected cells (B) and 50 ng/mL SCF in MegaCult-C medium. (C) and (D) are images of CFU-E cultured in 3 U/mL rhEPO and 50 ng/mL SCF. They were stained positively for anti-glycophorin A. The CFU-E cultured in the supernatant collected from the nucleofected cells were also stained positively for anti-glycophorin A (E and F). For a negative control, Ab anti-TNP was used and the CFU-E did not pick up any red stain at all (G and H).*

expression reached its highest levels on days 6 and 2, with 2683.76 mU/mL and 373.53 mU/mL, respectively.

In another two concomitant studies using the source plasmid to deliver the EPO gene into human MSC, it was found that the expression rose up to 4779.40 and 3965.35 mU/mL/1.0 μg pMCV-EPO/1×10^5 cells on day 1 post-nucleofection. In the first study, the EPO level continued to increase to 5147.45 mU/mL on day 6 and then fell sharply to 28.53 mU/mL on day 22. The EPO level never rose again and continued to show an expression level less than 10 mU/mL until 3 months later. In the second plasmid study, the EPO expression dropped to 2.45 mU/mL on day 22 and thus we stopped monitoring. Meanwhile, the MIDGE-EPO-transfected cells showed a fluctuating expression of EPO and maintained a relatively higher EPO secretion than that of plasmid-transfected cells. One of the MIDGE-EPO-transfected cells was found to express 641.56 mU/mL EPO even after 90 days of nucleofection (Figure 4). The EPO protein expressed by both MIDGE-EPO- and pMCV-EPO-transfected cells was able to induce the differentiation of HSC into erythroid colonies (Figure 5).

Previously, MIDGE vectors have been reportedly used mostly for enhancement of immunization and vaccination. Leutenneger *et al.* [20] coated MIDGE constructs encoding inactivated feline immunodeficiency virus (FIVgp140) alone or with feline IL-12, IL-16 or a CpG motif on gold microcarriers, and intradermal co-injection into the feline groups showed positive humoral and cellular immunity towards FIV. In another experiment [21], MIDGE vectors were used to construct a vaccine to combat Leishmaniasis in mice models. MIDGE were inserted with the coding sequence of LACK/p36, an Ag highly conserved among related *Leishmania* species. The results showed that COS-7 cells transfected with MIDGE encoding LACK/p36 expressed lower Ag compared with cells transfected with its source plasmid. However, when the constructs were injected intradermally twice in the back of BALB/c mice, the first was found to confer similar protection (i.e. reduced thickness of the infected collateral footpads) with half the amount of DNA used compared with the latter. Lopez-Fuertes *et al.* [21] suggested that the Ag load may not be the only limiting factor when vaccinating with DNA and that MIDGE vectors have an unknown feature that makes them immunologically more efficient. Alternatively, it could be that MIDGE vectors induce a higher protein expression *in vivo* or that the amount of Ag expressed after two doses is above the threshold required for the induction of an immune responses [21]. A similar result has been achieved by Moreno *et al.* [22]. They showed that MIDGE encoding hepatitis B virus Ag (HBsAg) was capable of expressing Ag levels comparable with those obtained with plasmids over a range of doses of DNA injected intramuscularly into BALB/c mice. However, when they were injected intradermally MIDGE could surprisingly trigger higher amounts of Ab compared with the plasmid [22].

Our study has revealed that MIDGE vectors are more effective and useful than the corresponding plasmid (pMCV1.2) in long-term gene expression using nucleofection as a mode of gene transfer into human MSC, despite the high initial expression shown by pMCV1.2-transfected cells. Currently there is a lack of data on the stable expression of MIDGE *in vitro*, and the possibility of MIDGE-driven integration of genes into the genome of MSC should not be ruled out in this study. It is also not clear to us why the pMCV-transfected cells had a higher initial expression than the MIDGE vector-transfected cells, as the MIDGE had a 1.79-fold reduced size compared with its source plasmid [23]. The decline of the EPO concentration observed could be attributed to the progressive loss of plasmid as a result of endonuclease degradation. The plasmid could be linearized and might have adverse effects on the processing of uninjured and functional forms [24]. In this case, MIDGE seems to be more superior than pMCV as it lacks unnecessary genes that might trigger endonuclease degradation. The discrepancy in gene expression might also be because of the different molarity of the expression cassette as the same amount of DNA was used for nucleofection.

There have been a number of reports that have used viral vectors carrying the EPO gene for transduction into MSC [25–27]. The results showed that the hematocrit levels were increased in mice models even after 2 months. In another study, the plasma EPO was recorded as 67 mU/mL after a 14-day subcutaneous transplantation with collagen-embedded EPO-secreting marrow stroma, which released *c.* 3 U EPO/10^6 cells/24 h, and this level dropped to 15 mU/mL at day 147. Meanwhile the hematocrit levels had increased to 81% at 22 days following implantation with 10^7 collagen-embedded EPO MSC, and remained at values of 82–88% until day 106, surpassing 70% up to day 203 [28]. Although the results are promising, we should not rule out the risk of oncogene activation and the

immunotoxicity that the viral vectors might pose. Our results show that the MIDGE vector could be an alternative to viral vectors in gene transfer as MIDGE-EPO-transfected MSC could stably express EPO at even 3 months post-nucleofection and MIDGE abolish the transfer of therapeutically detrimental sequences.

In conclusion, our results demonstrate that human MSC can carry MIDGE vectors and express the EPO gene stably *in vitro*. MSC seem to work well synergistically with MIDGE vectors compared with the corresponding plasmid to deliver EPO continuously and efficiently. However, further study could be done to improve the gene expression level, for example to add a nuclear localization signal [29,30] or to improve the transfection method, perhaps by using magnetic transfection [31,32], and to look into the capability of transfected MSC to carry and deliver EPO in an animal model.

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